INTRODUCTION

The candidate oncogene ZNF217 (previously designated *ZABC1*), predicted to encode alternately spliced Kruppel-like transcription factors, was originally identified based on its core location in an amplicon on chromosome 20q13.2 in breast cancer cell lines and primary tumors, and its recurrent pattern of expression in tumors (1). 20q amplification, common in many human cancers, is also associated with overcoming senescence and p53-independent genome instability in cultured human uroepithelial cells (2, 3). The current experiments were initiated with the aim of determining which specific cellular functions altered by ZNF217 overexpression may be responsible for causing selection of cells with increased gene copy number and tumor progression. Several cell biological assays useful in distinguishing normal HMEC from immortally and tumorigenically transformed cells have been used to compare ZNF217-transduced cells with control cells. Alteration of specific phenotypic properties in HMEC overexpressing ZNF217 will provide direct evidence of the gene's oncogenic potential, and provide information about the biochemical pathways affected. Such information should be valuable in evaluating the prognostic implications of ZNF217 overexpression, and in designing new therapeutic strategies to combat the effects of such overexpression.

BODY

Technical Objective 1: Express ZNF217 cDNA in normal and immortalized HMEC.
The coding sequence of ZNF217 was subcloned into a standard, widely used retroviral vector, LXSN (4), for efficient uptake and expression in HMEC. High titer amphotropic stocks of ZNF217 and control retrovirus were prepared using a transient packaging system (5) and used to infect recipient HMEC cultures. Northern analysis showed clear evidence of ZNF217 mRNA overexpression, and immunoblot analysis using a crude antibody preparation showed evidence of ZNF217 protein expression, in the infected cells. A separate experiment employing a ZNF217-EGFP fusion construct in a plasmid vector showed preferential nuclear localization of fluorescent signal in transfected COS7 cells, confirming the presence of a functional nuclear localization signal in the ZNF217 sequence. Interestingly, the ZNF217-EGFP fusion protein exhibited a particularly punctate, speckled pattern of fluorescence in some nuclei.

Technical Objective 2: Determine whether ZNF217 expression influences the growth rate of adherent normal HMEC.
Experiments were conducted in finite lifespan HMEC that had or had not received exposure to a chemical carcinogen prior to ZNF217 transduction. Growth rates in complete growth medium, in the absence of EGF, or in the presence of TGFβ were compared in cells transduced with ZNF217 or control virus (LXSN). In each case, no statistically significant differences in growth rates were noted (data not shown).

Technical Objective 3: Determine whether ZNF217 expression extends the replicative lifespan of or immortalizes normal HMECs when expressed alone or in combination with viral oncogenes.
In five independent experiments (see Nonet et al., Cancer Research 61: 1250-1254, 2001 in Appendix 1 for details), ZNF217-transduced cultures maintained growth beyond the point where control cells senesced. HMEC that overcame senescence initially exhibited heterogeneous growth and continued telomere erosion, followed by increasing telomerase activity, stabilization of telomere length, and resistance to TGFβ growth inhibition. This pattern is similar to what we have observed in rare HMEC lines immortalized following exposure to a chemical carcinogen, where telomerase reactivation and attainment of good uniform growth occurred in an incremental, apparently epigenetic manner, a process we have termed "conversion," as a consequence of overcoming senescence.

The data demonstrate that constitutive aberrant expression of ZNF217 can immortalize finite lifespan HMEC. However, the precise frequency of immortalization has not yet been determined. Southern analysis of retroviral integration sites in ZNF217-transduced HMEC growing past senescence suggested that these cultures were rapidly overgrown by distinct clonal populations. In an effort to determine whether distinct chromosomal alterations might be conferring growth advantages on clones immortalized with ZNF217, DNA from three different immortalized cultures was used for quantitative measurement of DNA copy number using comparative genomic hybridization (CGH) (6). CGH analysis showed low level regional DNA-sequence copy number variations on chromosomes 1q and 8q common to all three cell lines. The region amplified on 8q included the c-myc oncogene, which itself has been shown to cause HMEC immortalization when overexpressed (7). In addition, each line showed unique regions of high and low level DNA-sequence copy number variations. These sites of regional copy number variation, some of which have also been frequently observed in breast cancer cell lines and primary tumors (6), may contain genes that cooperate with ZNF217 in facilitating growth and immortalization.

Technical Objective 4: Determine whether ZNF217 expression influences the ability of immortalized HMEC to grow under anchorage independent conditions.
Three independently derived ZNF217-immortalized HMEC lines were assayed for anchorage-independent growth by suspension of single cells in methylcellulose and incubation on PolyHEMA-treated plates for four weeks. Colony forming efficiency was found to be < 0.03% for all three lines (data not shown). Moreover, the cells in the few colonies obtained were not enriched for anchorage independence when re-analyzed. This data indicates that although ZNF217 can promote immortalization of HMEC, it does not confer anchorage independence.

Technical Objective 5: Determine whether ZNF217 expression affects growth/differentiation/invasion response to extracellular matrix.
These experiments have not yet been performed.

Technical Objective 6: Determine whether ZNF217 expression influences the ability of HMEC to undergo apoptosis when forced to express adenovirus E1a.
These experiments have not yet been performed.

Technical Objective 7: Determine whether ZNF217 expression alters HMEC tumorigenicity in nude mice.
Three independently derived ZNF217-immortalized HMEC lines were assayed for tumorigenicity in nude mice and SCID mice. For the nude mice, 1.0×10^7 cells per mouse were injected along with Matrigel into 9, 10, and 8 nude mice respectively. Although in each case, the cells initially formed palpable lumps at two weeks after injection, all the lumps started to regress by week 3. By 40 days, there were no signs of tumor growth. For the SCID mice, 2.0×10^7 cells per mouse were injected along with Matrigel into 5 animals each. The SCID mice also showed palpable lesions soon after injection, but these lesions also regressed. We conclude from these experiments that overexpression of ZNF217, by itself, is not sufficient to confer tumorigenicity in normal or carcinogen-treated HMEC.

KEY RESEARCH ACCOMPLISHMENTS
• Overexpression of a retrovirally transduced ZNF217 gene in normal finite lifespan and carcinogen treated extended-life HMEC cultures leads reproducibly to immortalization.

• Immortalization of ZNF217-transduced HMEC occurred without changes in p53 inducibility or function, and without changes in Rb expression.

• Reactivation of telomerase and attainment of uniform good growth +/- TGFβ occur incrementally after ZNF217-transduced HMEC have overcome senescence.

• CGH analysis of three cell lines shows common low level regional DNA-sequence copy number variations on chromosomes 1 and 8 that may be sites of genes that cooperate with ZNF217 in facilitating growth and immortalization.

• Overexpression of ZNF217, by itself, is not sufficient to confer anchorage independence or tumorigenicity in HMEC.

REPORTABLE OUTCOMES

Nonet, G.H., Stampfer, M.R., Chin, K., Gray, J.W., Collins, C.C., and Yaswen, P. The *ZNF217* Gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. Cancer Res. 61: 1250-1254, 2001.

Stampfer, M.R. and Yaswen, P. Immortal transformation and telomerase reactivation of human mammary epithelial cells in culture, in: Advances in Cell Aging and Gerontology: Telomerase, Aging and Disease (M. Mattson and T. Pandita, eds.) Elsevier, Amsterdam, In press.

Abstract - G. H. Nonet, M.R. Stampfer, C.C. Colins, J.W. Gray, and P. Yaswen. Immortal transformation of human mammary epithelial cells following overexpression of ZNF217: a gene amplified and overexpressed in breast cancer. Proc. Amer. Assoc. Cancer. Res. 41, 318, 2000.

Abstract - P. Yaswen., G. H. Nonet, C.C. Collins, J.W. Gray, and M.R. Stampfer. Immortalization of human mammary epithelial cells by ZNF217: a novel gene amplified and overexpressed in breast cancers. Proc. DOD Breast Cancer Research Program Meeting I, 83, 2000.

Abstract - P. Yaswen, G. H. Nonet, C.C. Collins, J.W. Gray, and M.R. Stampfer. Human mammary epithelial cell immortalization by ZNF217: a novel gene amplified and overexpressed in breast cancers. Telomerase and Telomere Dynamics in Cancer and Aging June 24-28, 2000 San Francisco, CA.

Abstract - G. H. Nonet, M. R. Stampfer, K. Chin, J. W. Gray, C. C. Collins, and P. Yaswen. The ZNF217 gene amplified in breast cancers promotes immortalization of human mammary epithelial cells**.** Molecular Biology and New Therapeutic Strategies: Cancer Research in the 21st Century February 12-16, 2001 Maui, HI.

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CONCLUSIONS

The results obtained support the hypothesis that ZNF217 gene amplification is frequently found in breast cancers because it is involved in enabling breast cells to overcome the restraints of senescence, thus allowing the cells to continue growing and accumulating other changes necessary for malignant progression. The slow gradual changes in telomerase activity and growth in ZNF217-transduced cells after they have overcome senescence resemble the changes seen during the conversion process in carcinogen-immortalized HMEC, where measurable telomerase reactivation follows rather than precedes the overcoming of senescence. While viral oncogenes HPV E6 and E7 can also immortalize HMEC (8), HPV is not associated with most human cancers, other than those of the cervix. ZNF217 transduction, on the other hand, represents a biologically relevant model for one of the changes involved in immortalization and in cancer progression. Moreover, ZNF217 may prove to be a clinically useful marker as well as a novel therapeutic target.

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APPENDICES

1. Nonet, G.H., Stampfer, M.R., Chin, K., Gray, J.W., Collins, C.C., and Yaswen, P. The ZNF217 Gene amplified in breast cancers promotes immortalization of human mammary epithelial cells. Cancer Res. 61: 1250-1254, 2001.

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